FROM EBOLA VIRUS DISEASE TO BACTERIAL TOXICITY:

AN IN SILICO AND IN VITRO STUDY

Macías Martín, Minerva

INS Pompeu Fabra, Martorell

Supervisor: Dr. Francisco Pérez

Abstract

The ecotoxicity of seven different bacteria was investigated *in vivo* on the freshwater crustacean *Daphnia magna.* The effect of *Bacillus cereus, Bacillus megaterium, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens, Staphylococcus epidermis* and *Serratia marcescens* was tested according to ISO 6341 (2012) standard procedure. The most active bacterium has been studied using *in silico* methods to find possible target proteins.

Keywords: Toxicity; Ecotoxicity; Bacteria agents; *Daphnia magna.*

FROM EBOLA VIRUS DISEASE TO BACTERIAL TOXICITY:

AN IN SILICO AND IN VITRO STUDY

1. Introduction

Metchnikoff discovered phagocytosis through the infection of *Daphnia magna,* among other animal species, with spores of infectious fungus [(1)]. Our experimental design was inspired by Metchnikoff and seven different bacteria are used (*Bacillus cereus, Bacillus megaterium, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens, Staphylococcus epidermis* and *Serratia marcescens*) instead of infectious spores.

2. Materials and methods

2.1 Materials

2.1.1 Daphnia magna

The use of *Daphnia magna* (Figure 1) for this kind of experiments is largely extended because of its short lifespan and its fast-reproductive capabilities. Their transparent aspect allows the scientists to study the internal organs in live specimens. It was thanks to their transparency that Metchnikoff could see how phagocytes worked. Also, their thin membrane makes them perfect specimens to be used as a test species, since it allows testing compounds to be absorbed in order to know their possible toxicity. The *Daphnia magna* specimens were donated by CESIRE (Dept. Ensenyament, Generalitat de Catalunya).

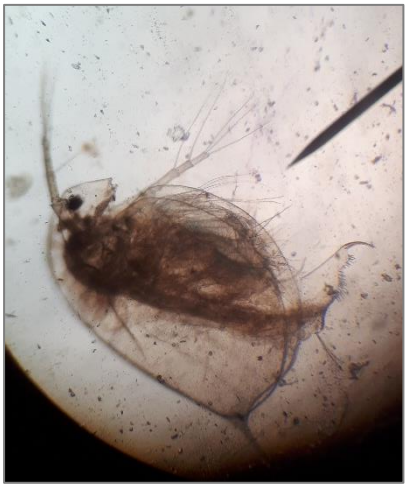


Figure 1. Daphnia magna specimen observed through a Zeiss microscope.

Figure 2. Different pots where the Daphnia specimens were living and reproducing from August to October 2017.

2.1.2 Media

The *Daphnia magna* used for the experiments described next have been living in pots (Figure 2) with mineral water at room temperature and receiving direct sunlight during the day. The mineral water used was *Aigua de Ribes* because of its low salinity and neutral pH (7.5).

2.1.3 Bacteria

The seven species of bacteria have been living in airtight crystal tubes which have been conserved at 5ºC. The seven bacteria were the following ones: *Bacillus cereus* Frankland and Frankland (ATCC14579), *Bacillus megaterium* de Bary (ATCC14581) (Figure 3), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC25922) (Figure 4), *Micrococcus luteus* (Schroeter) Cohn (ATCC4698), *Pseudomonas fluorescens* Migula (ATCC13525), *Staphylococcus epidermidis* (Winslow and Winslow) Evans (ATCC14990) and *Serratia marcescens* Bizio (ATCC27143).

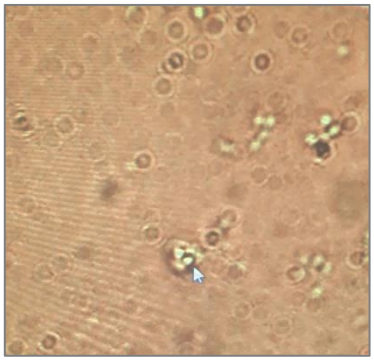


Figure 3. Bacillus megaterium (x630). Photograph taken through a Moticom camera connected to a Zeiss microscope in Universitat de Barcelona. The bacterium is possibly in a defense structure.

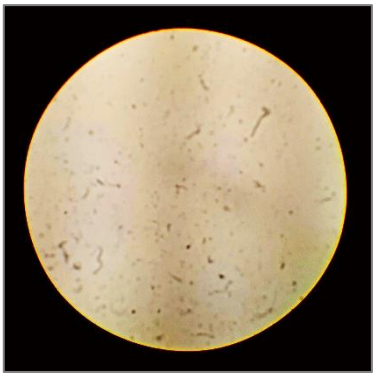


Figure 4. Escherichia coli (x 1000). Photograph done through a Zeiss microscope in Universitat de Barcelona.

2.1.4 Oxygen pump

An oxygen pump was used to keep the medium where the *Daphnia* where living oxygenated. The pump power is 2.2W and 50/60 Hz frequency. It was the model SE-302 by JAD Aquarium (Figure 5).

Figure 5. Air pump from JAD, model SE-302.

2.1.5 pH and its interface

A pH meter (Figure 6) was used to measure the acidity or basicity of the water in the different water tanks. The range of the pH meter went from 0 to 14 points of acidity and its accuracy was ±0.2 pH units. The pH meter needed an interface to compile the data and show it on a screen. The interface used on this study was the LABQUEST mini by Vernier (Figure 7). Its maximum sampling rate is 100 kHz.

Figute 6. pH sensor pH-BTA by Vernier.

Figure 7. Side view of the interface LABQUEST mini by Vernier.

2.1.6 Swissdock software

The Swissdock software by the Swiss Institute of Bioinformatics [(2)] have been used to study the different interactions between the *Daphnia* and the bacteria.

2.1.7 R programming language and RStudio

R professional statistical software was used to analyse the data arising from the experiments and the *in silico* procedures. The libraries used on this project were “nortest”

2.1.8 Zeiss microscope

A Zeiss microscope was used to observe the bacteria and the *Daphnia magna* specimens. The microscope was from Universitat de Barcelona.

2.1.9 Moticom camera

A Moticom camera (Figure 8) was used to increase the size of what was observed through the microscope and project it to a white screen.

Figure 8. Moticom camera connected to a microscope and to the projector.

2.2 Methods

2.2.1 Growing medium

Figure 9. *Two tubes used on a Serratia marcescens experiment. The left one, only containing water and 20 Daphnia, was the control one. The right one, with a reddish tone, contained 2 ml of Serratia and 20 Daphnia.*

The original specimens which were donated by CESIRE (Dept. Ensenyament, Generalitat de Catalunya) were separated in three different pots. One of them was only for the *Daphnia* specimens' reproduction, which means that no specimens where chose for the experiments if they were on this pot. When new specimens were born, these were transferred to one of the other two pots. Each day, the pump provided oxygen to the pots for 5 minutes. Every 5 days the *Daphnia* were fed with a pinch of crushed dog's food. When the water started being crystal clear, a nutritive juice was poured into the pots. This juice was made of water and green lettuce leaves which were first crushed.

2.2.2 ISO

ISO 6341 (2012) specifies a method for the determination of the acute toxicity to *Daphnia magna.* The official document is not public. This method specifies the water quality through its dissolved oxygen and pH, the control batch, the control solution, what is an immobilization, EC_{50}, neonates (<24 hours old) and the test batch. Another similar method that could be suitable for this kind of experiment would be the LD_{50} (median lethal dose). It calculates what dose is required to kill the 50% of the tested population a specified time.

2.2.3 Bacterial experiments

Two test tubes were used on each experiment (Figure 9). One of them contained 2 ml of a certain bacterium and 18 ml of water which contained 20 *Daphnia* (9:1 propotion), which is a high concentration of bacteria. The mix was in a crystal test tube for 24 hours. Next to this test

tube, another one was placed with 20 *Daphnia* in 20 mL of water as a control which showed if *Daphnia* were dying because of the bacteria or other external factors as the temperature or the concentration of oxygen. After that time, the living *Daphnia* in both tubes were counted. If more than two *Daphnia* from the control test tube were dead, the experiment was considered void. We carried out three experiments for each bacterium (a total of 21) in order to ensure that the results were reliable.

2.2.4 Heart beat experiment

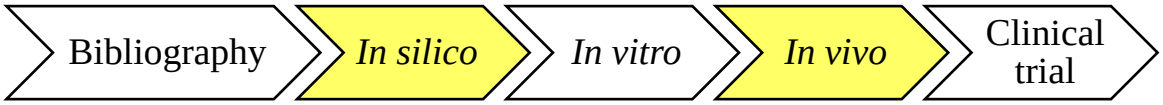
A procedure designed by the Nuffield Foundation was followed:

- **Investigating factors affecting the heart rate if *Daphnia*:**

1. Take a small piece of cotton wool, tease it out and place it in the middle of a small Petri dish.
2. Select a large *Daphnia* and use a pipette to transfer it onto the cotton wool fibres.
3. Immediately add pond water to the Petri dish until the animal is just covered by the water.
4. Place the Petri dish on the stage of a microscope and observe the animal under low power. The beating heart is located on the dorsal side just above the gut and in front of the brood pouch (see diagram). Make sure that you are counting the heart beats, and not the flapping of the gills or movements of the gut. The heart must be observed with transmitted light if it is to be properly visible.
5. Use a stopwatch to time 20 seconds and count the number of heart beats in several periods of 20 seconds. The heartbeat of *Daphnia* is very rapid, so count the beats by making dots on a piece of paper in the shape of a letter S. Count the dots and express heart rate as number of beats per minute.
6. At the end of the investigation, return the *Daphnia* to the stock culture.

2.2.5 The nature of our experiments

Our experiments were followed *in silico* and *in vivo* procedures.



3. Results

Daphnia's habitat must have a pH between 6.5 and 7.5 and a salinity below the 5% of seawater, that is to say below 1500mg/L (5), so the chosen water fitted perfectly the conditions where *Daphnia* could live. To make sure that the pH on the tanks was the optimum, we used the pH meter. The outcomes for the three water tanks showed an arithmetic mean of ($\bar{x}$): 7.55 and a standard deviation of (s): 0.141 (almost no variation between the outcomes) (Table 1). These data were calculated using RStudio.

	Measure 1	**Measure 2**	**Measure 3**	**Total**
Mean	7.55	7.72	7.83	7.55
Standard deviation (SD)				0.1410674

Table 1. Outcomes of RStudio concerning the mean and the standard deviation of the pH points of the three water tanks.

The results of the 21 experiments carried out from august to October 2017 were noted on a table and as it can be seen *Serratia marcescens* showed the highest mortality among all the bacteria (Table 2, Figure 10).

Bacterium	**Nº of *Daphnia* at the starting point**	**Living *Daphnia* after 24 hours. (1st experiment)**	**Living *Daphnia* after 24 hours. (2nd experiment)**	**Living *Daphnia* after 24 hours. (3rd experiment)**	**Average percentage of mortality (%)**
Bacillus cereus	20	17	15	13	25%
Bacillus megaterium	20	8	11	9	53%
Escherichia coli	20	8	9	8	58%
Micrococcus luteus	20	0	3	5	87%
Pseudomonas fluorescens	20	10	9	11	50%
Staphylococcus epidermidis	20	8	10	7	58%
Serratia marcescens	20	0	0	0	100%

Table 2. Mortality rate of the 7 different bacteria after 3 experiments for each one. The three infections started with 20 living Daphnia. In red, mortality rates above 75%.

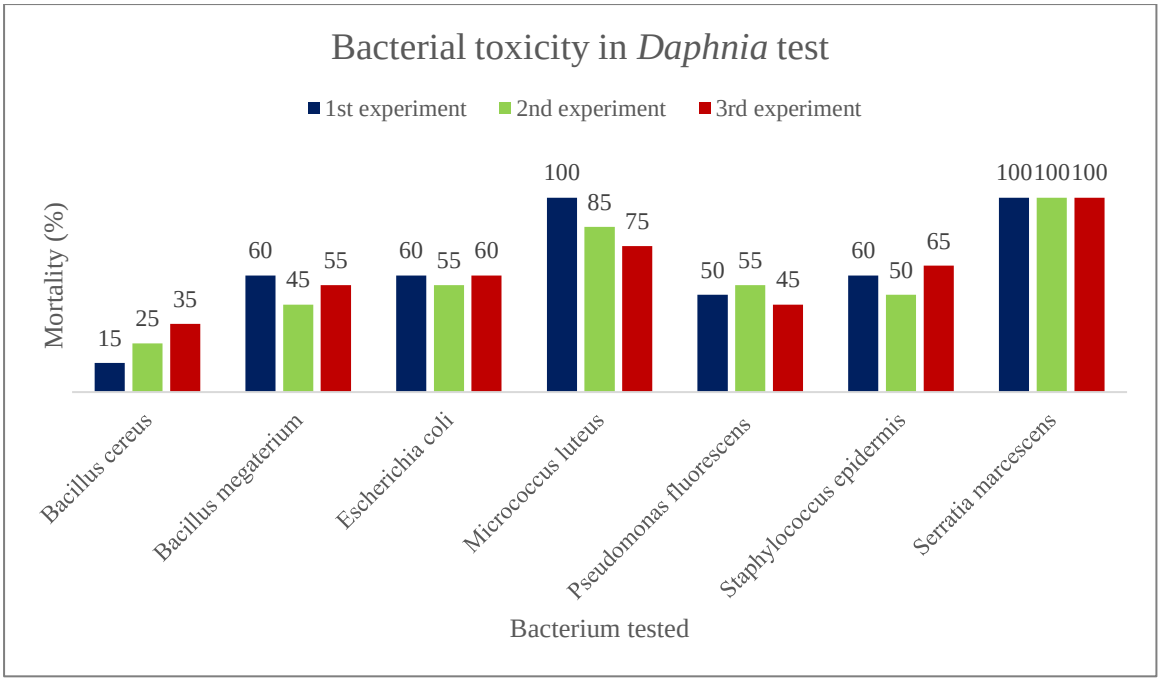


Figure 10. Representation of the mortality in three different experiments for each bacterium. All the cases were done with 20 individuals of Daphnia magna, at room temperature with direct sunlight during the day.

In view of the high mortality caused by *Serratia marcescens* and how well studied this organism is, we decided to do a deeper study of the interaction between *Daphnia magna* and this bacterium. The main hypothesis was that the different chitinases of the *Serratia marcescens* could destroy the chitin wall of the *Daphnia magna*. This wall has also N-acetylmuramic acid, also known as MurNAc. The cellular wall separates the external medium of the *Daphnia*'s internal medium. The breakage of this wall would cause an immediate death.

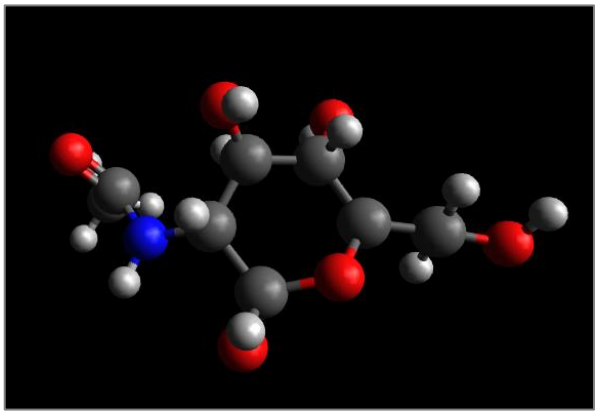


Figure 11. Structure of GlcNAc seen in AVOGADRO software following the molecule's isomeric smiles posted on PubChem. The grey dots represent carbon atoms, the red dots represent oxygen atoms and the blue dot represents a nitrogen atom.

Chitin is a derivative of glucose described as a long-chain polymer of N-acetylglucosamine (GlcNAc) (Figure 11). It is often presented as vast networks of covalently bonded carbohydrates with hydrogen bonds packing the different layers [(3)].

Serratia marcescens chitinolytic machinery is based on chitinases, a chitin-binding protein and a chitobiase (Figure 12). *Serratia marcescens* major chitinases are ChiA, ChiB [(4)] and ChiC, the last one being divided in ChiC1 and Chi C2 [(5)], which work altogether with a chitobiase [(6)] and a chitin- binding protein named CBP21 [(7)]. ChiA and Chi B are processive chitinases which move along chitin chains in opposite directions. ChiA degrades the GlcNAc polymers from their reducing ends while ChiB does the same from the non-reducing ends. Both enzymes give as a result of their process of degradation chitobiose (dimers of chitin, i.e. (GlcNAc)2). ChiC is an endo-acting non-processive chitinase [(5)] which makes random cuts, opening different regions and letting ChiA and ChiB act. These cuts are made on the most amorphous regions of the polymer. The chitin binding protein 21 (CBP21) introduces chain breaks by oxidative cleavage in the most crystalline regions yielding aldonic acids to the GlcNAc, transforming it to GlcNAcA. Lastly, *Serratia marcescens'* chitobiase (N-Acetylglucosaminidase) [(8)] converts chitobiase and short chito-oligosaccharides to monomers. These would be the theoretical way that *Serratia marcescens* uses to destroy *Daphnia magna's* cell wall.

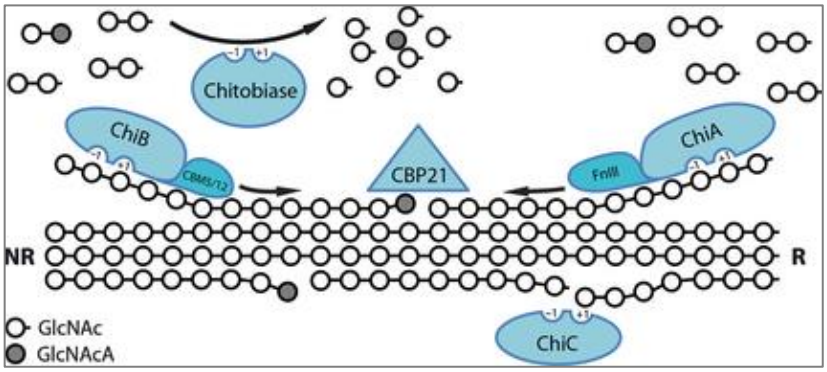


Figure 12. Serratia marcescens' chitinolytic machinery. The figure shows how ChiB degrades chitin from the non-reducing ends (NR), while ChiA is located on the reducing ends (R). ChiC and CBP21 are shown cutting the chitin chains. Chitobiase is placed on the top, converting dimers to monomers.

All the chitinolytic machinery is shown in the next page table with their respective Uniprot and Protein Data Bank IDs for further online information (Table 3).

Molecule	Family	Uniprot ID	PDB ID	Resolution (Å)	Method
Chitinase A	GH-18	P07254	1CTN	2.3	X-Ray diffraction
Chitinase B	GH-18	Q54276	1E15	1.9	X-Ray diffraction
Chitinase C	GH-18	Q700B8	4AXN	1.68	X-Ray diffraction
Chitobiase	GH-20	Q54468	1QBA	1.85	X-Ray diffraction
CBP21	CBM33	O83009	2BEM	1.55	X-Ray diffraction

Table 3. Families and IDs of the chitinolytic machinery of Serratia marcescens. Resolution and Method information is about the PDB entries.

With the online IDs, the *in silico* part of the study was started. With the Swissdock software [(2)], the links between chitotriose and the different chitinases could be easily seen. The interactions between Chitobiose and Chitobiase were performed as well. (Figure 13). The lower binding energy was the one by chitobiase (15.39 kcal/mol), which is statistically lower than the one showed by Chitinase B (18.5 kcal/mol) according to U Mann Whitney test performed with RStudio.

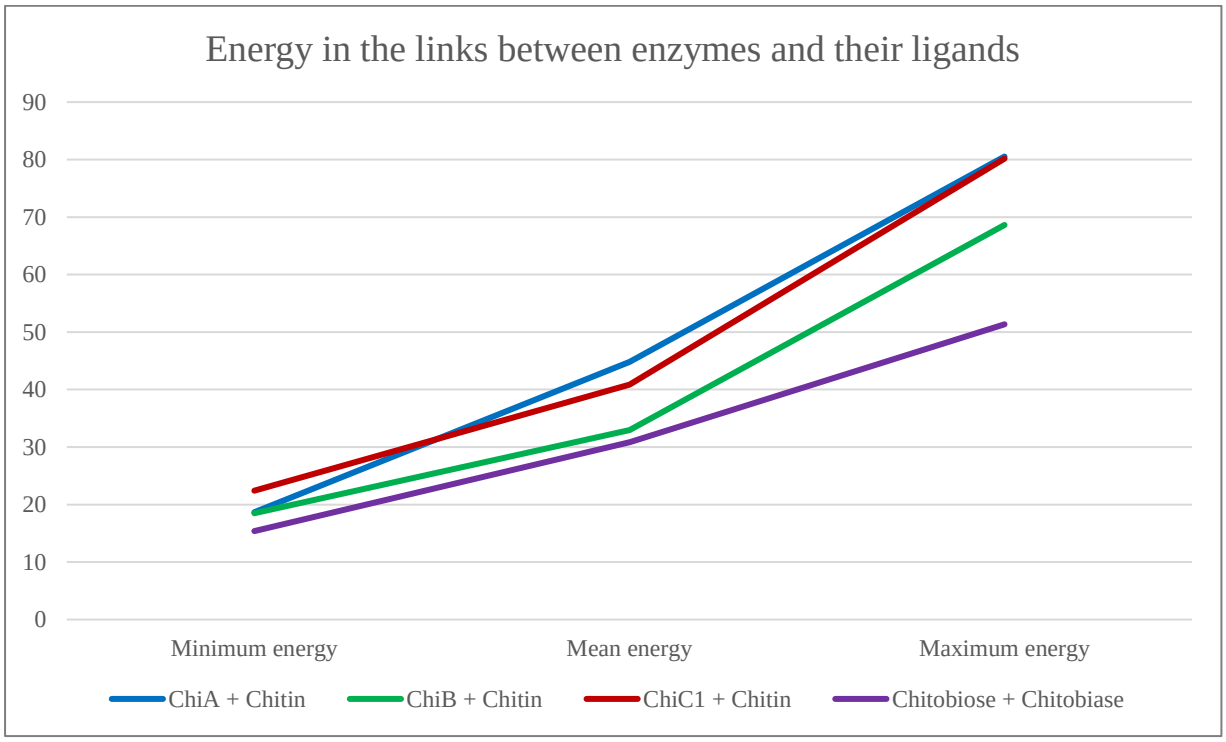


Figure 13. Outcomes of SwissDock for the assessment of the interactions of Chitotriose with Serratia marcescens' chitinases A, B and C1 and Chitobiose with Serratia marcescens' chitobiase. The data was analysed with RStudio.

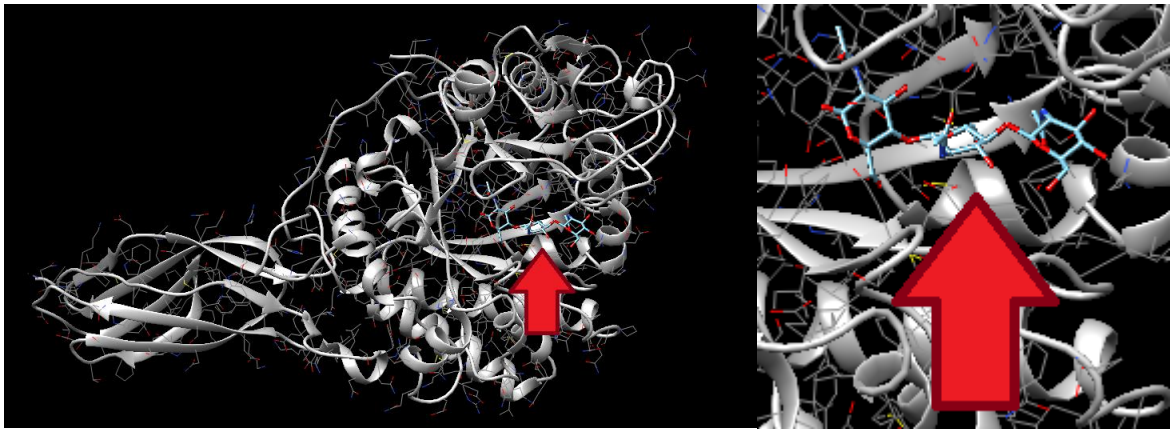


Figure 14. Above, docking outcome for the Chitotriose and Chitinase A assessment by SwissDock by the Swiss Institute of Bioinformatics. Coloured in white, the Chitinase A from Serratia marcescens; coloured in blue and signalled with a red arrow, the chitotriose molecule.

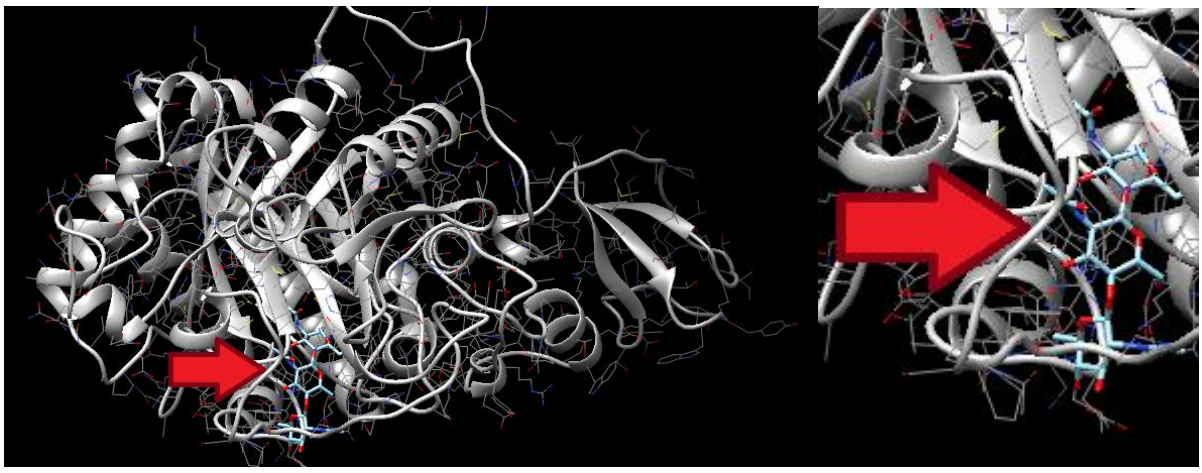


Figure 15. Above, docking outcome for the Chitotriose and Chitinase B assessment by SwissDock by the Swiss Institute of Bioinformatics. Coloured in white, the Chitinase B from Serratia marcescens; coloured in blue and signalled with a red arrow, the chitotriose molecule.

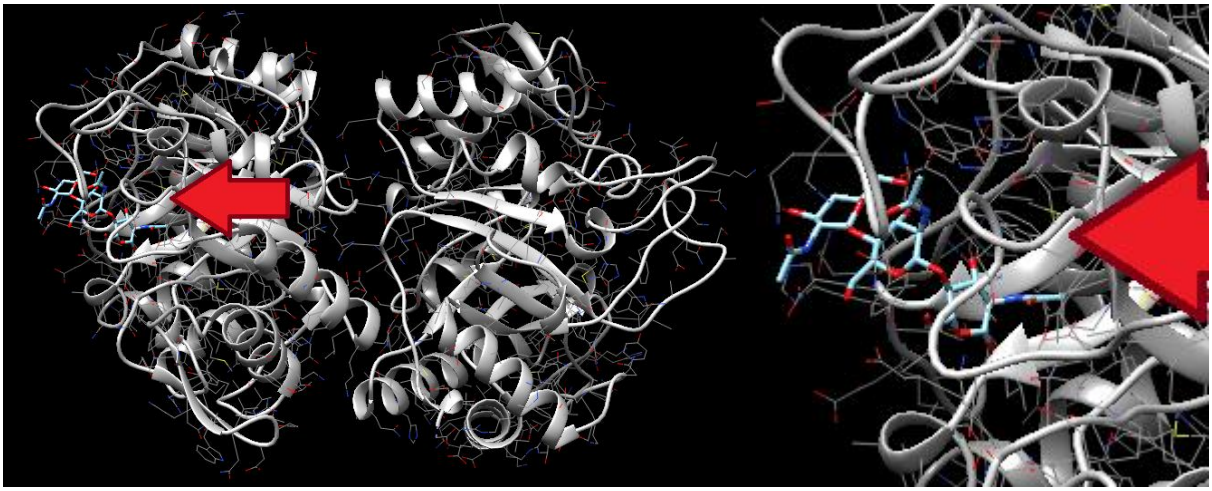


Figure 16. Above, docking outcome for the Chitotriose and Chitinase C1 assessment by SwissDock by the Swiss Institute of Bioinformatics. Coloured in white, the Chitinase C1 from Serratia marcescens; coloured in blue, the chitotriose molecule.

The links between the chitobiase and the chitobiose showed the lower energy. This could be because the chitobiose is more degraded, in fact it is dimers of chitin, when it interacts with the chitobiase. In contrast, the different chitinases have to interact with long chains of chitin, not only dimers. This previous degradation of the chitobiose may make the connection with its enzyme easier and more stable. It is remarkable the similarity between the energy showed in ChiA and ChiC1 docks despite their distinct functions in the chitinolytic machinery of *Serratia marcescens.*

Docking	**Shapiro-Wilk normality test**	**Anderson-Darling normality test**	**Cramer-von-Mises normality test**	**Kolmogorov-Smirnov normality test**	**Pearson normality test**	**Shapiro-Francia normality test**
ChiA + Chitin	$3.479 \cdot 10^{-8}$	$1.645 \cdot 10^{-12}$	$8.565 \cdot 10^{-9}$	$2.592 \cdot 10^{-9}$	$1.74 \cdot 10^{-11}$	$2.643 \cdot 10^{-7}$
ChiB + Chitin	$2.257 \cdot 10^{-7}$	0.001661	0.04013	0.07359 *	$9.495 \cdot 10^{-5}$	$9.486 \cdot 10^{-7}$
ChiC1 + Chitin	$2.811 \cdot 10^{-9}$	$1.468 \cdot 10^{-10}$	$4.886 \cdot 10^{-8}$	$9.853 \cdot 10^{-7}$	$2.221 \cdot 10^{-15}$	$2.439 \cdot 10^{-8}$
Chitobiase + Chitobiose	$5.57 \cdot 10^{-5}$	$2.997 \cdot 10^{-5}$	$7.774 \cdot 10^{-5}$	$3.096 \cdot 10^{-6}$	$1.506 \cdot 10^{-12}$	0.0001317

Table 4. Results of the different normality tests performed by RStudio with the data obtained from the SwissDock assessment. In black, results confirming that the Energy data has a non-normal distribution. In red, results confirming the normal distribution of the Energy data. A p-value under 0.05 means that data has a non-normal distribution. All these tests are made to distinguish what methods will be applied later depending on their kind of distribution.

The outcomes of the different normality tests performed by RStudio mainly show that the data has a non-normal distribution (Table 4). Only the Kolmogorov-Smirnov test show a normality distribution on the ChiB+Chitin interaction. Below this text, the boxplots of the interactions between the enzymes and the substrates can be seen. The points outside the box represent extreme energies and the line inside the box show the arithmetical mean. As can be seen, more remarkable on the ChiA and Chitobiase, this means are displaced from the middle of the box. This is a clear sign of a non-normal distribution of the data.

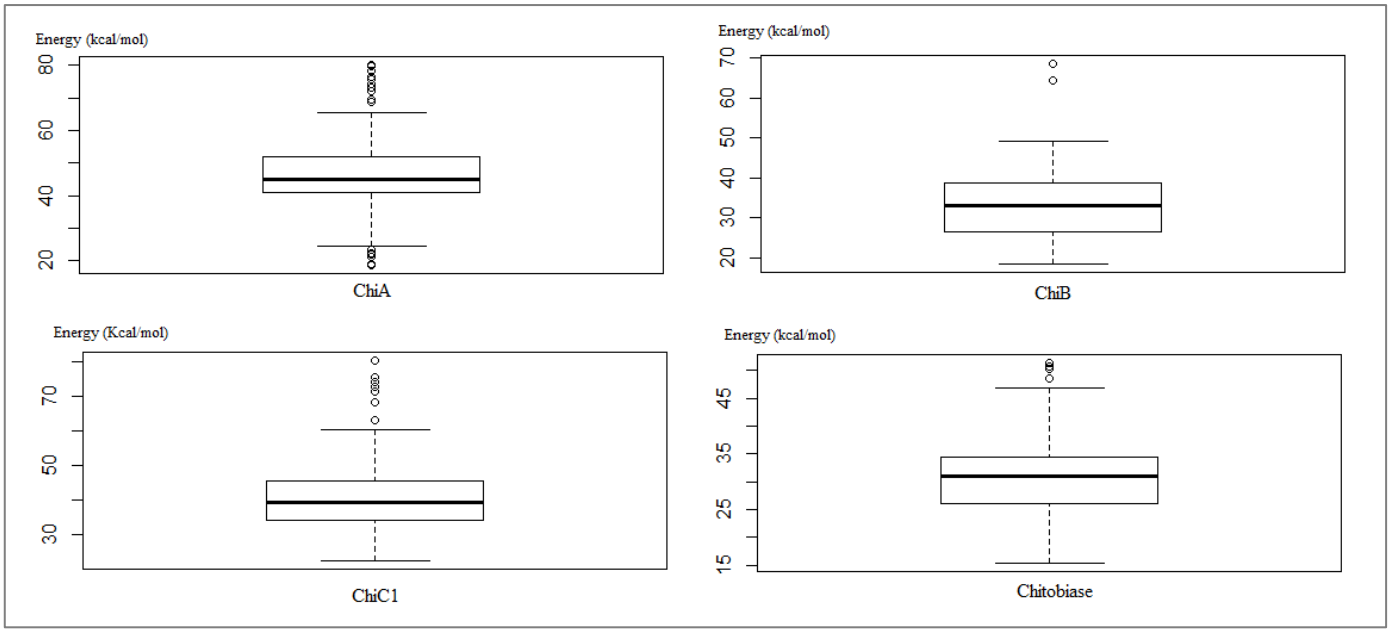


Figure 17. 4 different boxplots showing the distribution of the different interaction's energy between the chitinases and the chitin, and the chitobiase with the chitobiose (RStudio).

Another way to bolster this hypothesis is to plot the data creating a histogram. If the data follows a normal distribution, the plot should be similar to the Gaussian Bell curve. As it can be seen on the histograms below, none of our data sets follow the normal data distribution.

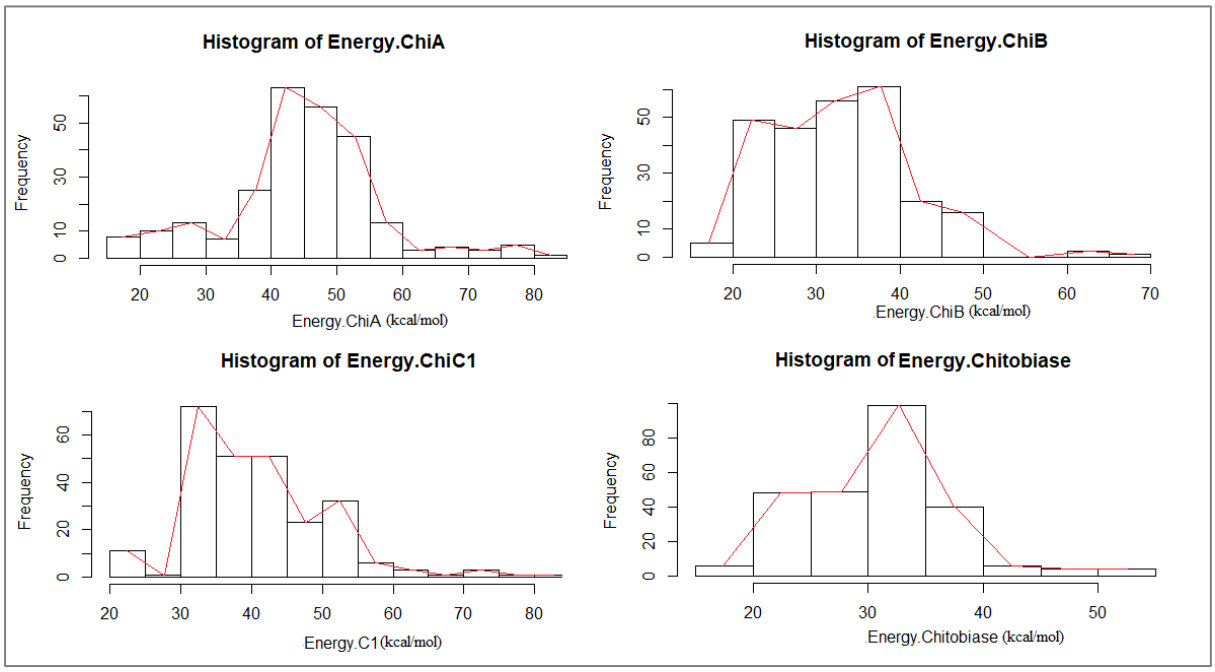


Figure 18. Histograms for the different docking energies between enzymes and chitotriose. (RStudio).

Once we have ensured that the data follow a non-normal distribution, we proceed to test the data with the appropriate statistical method (Figure 19).

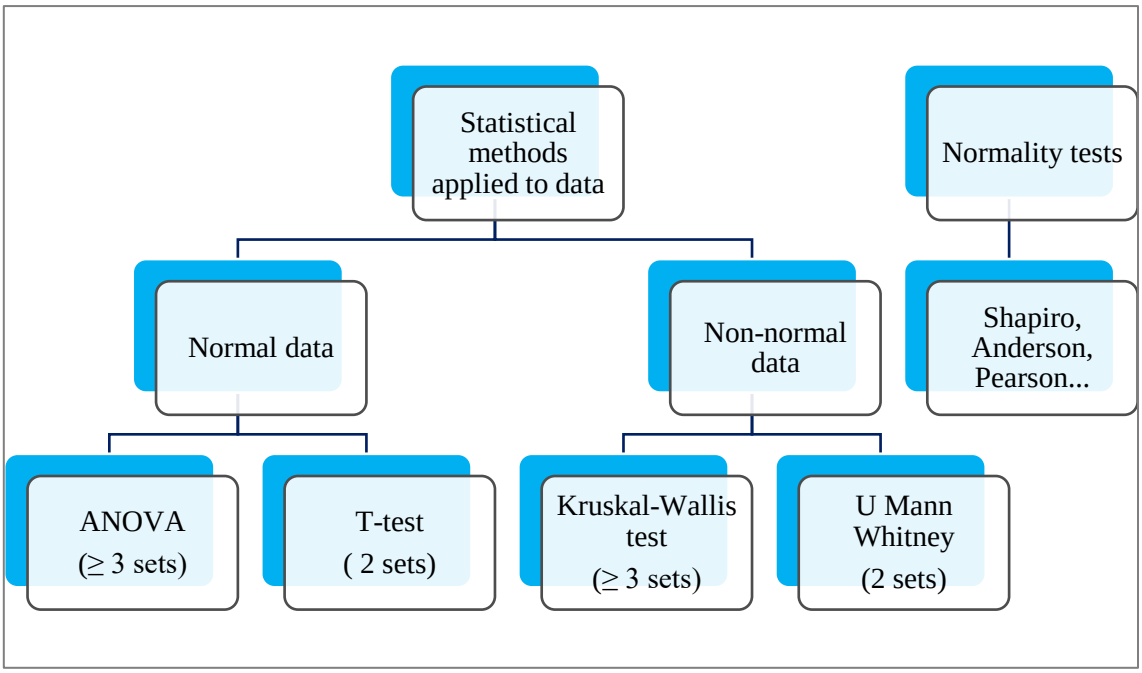


Figure 19. Statistical methods applied to data obtained in this research project and some of the multiple normality tests used.

Knowing that our 4 samples (Energies from Chitinases A, B and C1's interactions with chitin and energies from Chitobiase's interactions with chitobiose) follow a non-normal distribution, we proceed to study them with the Kruskal-Wallis test.

This test is designed to differentiate if the samples come from populations with equal or different medians. The p-value will be fixed at $2.2 \cdot 10^{-16}$, that is to say that there is a $2.2 \cdot 10^{-16}$% probability of the test being wrong. The degrees of freedom in this study are 3 (df= (n-1) = 4-1 = 3). Degrees of freedom are the number of values in a study that have the freedom to vary [9]. Looking for an p-value=0.05 and df=3 on the Chi-Square table, our rejection region is R=7.815. Since it is observed that 321.063>7.815 (H>R), it is then concluded that the null hypothesis is rejected. That means that at least two sets of data have not the same median (Figure 20).

```
> kruskal.test(Chitinases)

        Kruskal-Wallis rank sum test

data:  Chitinases
Kruskal-Wallis chi-squared = 182.13, df = 2, p-value < 2.2e-16

> kruskal.test(ALL)

        Kruskal-Wallis rank sum test

data:  ALL
Kruskal-Wallis chi-squared = 321.06, df = 3, p-value < 2.2e-16
```

Figure 20. Kruskal-Wallis test results performed with R software and RStudio. The outcomes from the Chitinases test are shown in the top of the picture while the outcomes from the Chitinases+Chitobiase test are shown in the bottom. Kruskal-Wallis chi-squared value equals to H in the explanation of the test.

To a more specific study, the Kruskal-Wallis test is repeated only considering the Chitinases

interactions with chititriose (Figure 20). This time, the degrees of freedom will be 2 (df = (n-1) = 3-1 = 2). On the Chi-square table, df=2 and α=0.05 equals to R=5.99. It can be observed that our H is 182.12 and 182.12>5.99 (H>R), it is then concluded that the null hypothesis is also rejected on this test. It is concluded that our data sets come from populations with medians that are not equal.

Following the hypothesis where the *Serratia*'s chitinases would be able to destroy the *Daphnia*'s carapace and therefore the animal would not be able to keep its internal medium from the external environment, the next step was checking if this change on the animal physiology could alter the heartbeat of the *Daphnia*. To test this possible consequence of the infection of *Daphnia* with *Serratia marcescens* a procedure designed by the Nuffield Foundation was followed [(10)].

The outcomes of the heart rate study were noted on a table taking into account the means on the heart beats of 4 different specimens.

***Daphnia*'s health condition**	**Mean of the heart beats counted on 4 different specimens**				**Mean**	**Standard deviation**
Healthy *Daphnia*	200	202	198	195	199	2.58
Infected with *Serratia marcescens*	177	186	174	183	180	5.48

Table 5. Difference in the heart beats of healthy Daphnias magna and infected Daphnias magna at a temperature of 20ºC. The number of heart beats are counted in periods of 20 seconds and then multiplied by 3. The infected Daphnias were in contact with Serratia marcescens for 8 hours. We can observe that the mean was reduced from 199 beats/minute to 180 beats/minute, that is to say that the heartbeat decreased a 5.25%.

It can be observed that the mean was reduced from 199 beats/minute to 180 beats/minute, that is to say that the heartbeat decreased a 5.25%. That is a significant decrease taking into account that the bacterium had only 8 hours to take action. It would be reasonable to think that, in a larger period, the damage caused by the *Serratia* on the *Daphnia*'s exoskeleton would be able to produce such a decrease which would finally kill the animal.

To proceed with the statistical study, first the normality or non-normality distribution of the data has to be determined. Unlike with the chitinases and chitobiase docking, this time only Shapiro-Wilk and Pearson can be performed. For Anderson-Darling and Cramer-von-Mises normality test the sample has to be greater than 7; for Kolmogorov-Smirnov normality test the sample has to be greater than 4 and for Shapiro-Francia normality test sample size must be between 5 and 5000.

Data	Shapiro-Wilk normality test	Pearson normality test
Healthy *Daphnia* heart beat	0.9523	1
Infected *Daphnia* heart beat	0.7143	1

Table 6. Results of the normality tests performed with RStudio. All the results are higher than 0.05, what means that both sets of data have a normal distribution as their p-value is higher than p=0.05.

As it can be seen on Table 6, both sets of data have a normal distribution. Because of this kind of distribution and taking into account that there are only two sets of data, we proceed to perform a t-test to observe whether there are significant differences or not between the two sets (Figure 19).

Data	T-value	Degrees of freedom	P-value
Healthy and Infected *Daphnia* heart beat	6.0112	4.6386	0.002367

Table 7. Results of the Welch two sample t-test (RStudio).

As it can be seen, the p-value equals 0.002367, which is lower than 0.05. By conventional criteria, this difference is considered to be extremely statistically significant.

Figure 21. Daphnia magna lying in cotton wool. Image taken through an optical microscope. The darkest dot (1) is the compound eye of the animal and the brownish one (2), its heart.

The decrease in the heart rate is very sharped taking into account that the bacterium had only 8 hours to take action. It would be reasonable to think that, in a larger period, the damage caused by *Serratia* on the *Daphnia*'s exoskeleton would be able to produce such a decrease which would finally kill the animal.

4. Discussion

Previous studies have found *Serratia* on the guts of *Daphnia magna,* as a part of the animal's microbiota, along with a great proportion of *Limnohabitans* [10] and *Pseudomonas* [11]. The strain found (D33) has been already sequenced [12].

It seems like this symbiotic associations play an essential role in the *Daphnia*'s organism. In the absence of microbiota both the longevity and the population dynamics is affected, being both of them enhanced [10]. Lacking these bacteria on their guts, the population can be reduced up to 10-folds in comparison to a *Daphnia* population where each specimen has proper microbiota.

Other studies have looked for a relation between the absence of microbiota and the diet of the animals. It has been observed that the specimens with proper microbiota were always bigger than the ones who lacked it [13].

It seems contradictory that infecting *Daphnia magna* with a bacterium which has been found on its guts being part of its microbiota and playing this microbiota an essential role in multiple aspects of the life of the specimen (enhancing reproduction, the growth and the longevity), this infection would finally cause de death of the specimen in such a brief period (≥24 hours). However, the relationship between *Daphnia magna* and *Serratia marcescens* could be analogous to the one between *Vibrio cholera* and the host that this bacterium infects. *V. cholera* needs to be able to survive in the inside of its hosts in order to colonize them. It is usually found attached to the exoskeletons of zooplankton which are mainly made of chitin [14]. The bacterium uses the chitin of the exoskeleton as its sole source of carbon and uses it to grow and to obtain raw material. It would be logical to think that *Serratia marcescens* follows the same process. The high concentration presented on the mix with only 20 *Daphnia* would make that the bacteria gathered around the exoskeleton of the crustacean and started engulfing it. Thanks to its chitinolytic machinery, the bacteria

would finally break that mechanism of protection which lets the animal to separate its internal medium to the external one.

The Shell disease syndrome has been largely studied and it also supports our results. It consists on the erosion and pitting of the exoskeleton caused by bacteria, and sometimes fungi as well, which are able to destroy chitin [(15)]. It has been mainly studied in various kind of crabs (for example, the edible crab or *Cancer pagurus)* that have a much stronger carapace than *Daphnia magna.* It is expected that the breakage of the carapace of the *Daphnia* will be easier and faster and that the bacteria will damage the internal medium more easily. The main genera of bacteria which are strongly reported to cause that illness are *Vibrio, Aeromonas, Pseudomonas, Alteromonas, Flavobacterium, Spirillum, Moraxella, Pasteurella* and *Photobacterium* [(16)]. Being *Serratia marcescens* one of the most effective bacteria for degradation of chitin [(17)], it is logical to think that if other bacteria which are not as effective can degrade a crab exoskeleton, *S. marcescens* would have a fatal impact on *Daphnia's* carapace.

5. Conclusions

1. *Serratia marcescens* is the bacterium which causes the highest mortality on *Daphnia magna* (100%), being followed by *Micrococcus luteus* (87%), *Escherichia coli* (58%), *Staphylococcus epidermis* (58%), *Bacillus megaterium* (53%), *Pseudomonas fluorescens* (50%) and *Bacillus cereus* (25%).
2. *In silico* studies of docking energies of *Serratia marcenses* chitinases A, B, C and chitobiase using Swiss Bioinformatics Institute software showed the best binding energy is chitobiase with chitoriose (15.39 kcal/mol) and it statistically lower than chitinase B (18.5 kcal/mol) probably because chitobiose interacts with dimers of chitin while chitinases interact with whole and larger chains of chitin. It could also be because the chitinases pertain to different families.
3. This high mortality rate caused by *Serratia marcescens* is possibly due to its chitinolytic machinery which would be able to destroy the *Daphnia*'s exoskeleton. Without it, the animal could not keep its internal medium apart from the external environment. This change on the animal physiology could alter the *Daphnia*'s heartrate before causing its death (procedure designed by Nuffield Foundation).
4. *Serratia marcescens,* in 8 hours, produced a reduction on the heart rate on an infected *Daphnia* of 5.25% (from 199 beats/minute to 180 beats/minute) ($p<0.05$). This decrease, in a larger period, would be able to finally cause the death of the specimen.

6. Acknowledgments

Especial thanks to CESIRE for all the material provided and to ICE-FORCES and Dr. Francisco Pérez from the Universitat de Barcelona for letting us use their laboratory and their materials.

7. References

(1) : Tan S.Y, MD, JD and Dee M. K. 2009. Elie Metchnikoff (1845-1916): discoverer of phagocytosis. Singapore Med J; 50: 456.

(2) : SwissDock. Swiss Institute of Bioinformatics. [consulted: 14 august 2017]. Available at: http://www.swissdock.ch/

(3) : Suvamay Jana, Anne Grethe Hamre, Patricia Wildberger, Matilde Mengkrog Holen, Vincent G. H. Eijsink, Gregg T. Beckham, Morten Sørlie, and Christina M. Payne. 2016. Aromatic-Mediated Carbohydrate Recognition in Processive Serratia marcescens Chitinases. J. Phys. Chem. B 120, 1236-1249.

(4) : Jonathan D. G. Jones, Karen L. Grady, Trevor V. Suslow and John R. Bedbrook. 1986. Isolation and characterization of genes encoding two chitinase enzymes from Serratia marcescens. The EMBO Journal, 5:467-473.

(5) : K Suzuki, M Taiyoji, N Sugawara, N Nikaidou, B Henrissat, and T Watanabe. 1999. The third chitinase gene (chiC) of Serratia marcescens 2170 and the relationship of its product to other bacterial chitinases. Biochem J. 343: 587-596.

(6) : Hadar Kless, Yaron Sitrit, Ilan Chet andAmos B. Oppenheim. 1989. Cloning of the gene coding for chitobiase of Serratia marcescens. Mol. Gen. Genet. 217: 471-473.

(7) : Gustav Vaaje-Kolstad, Svein J. Horn, Morten Sørlie, Vincent G. H. Eijsink. 2013. The chitinolytic machinery of Serratia marcescens – a model system for enzymatic degradation of recalcitrant polysaccharides. The FEBS Journal. 280: 3028-3049.

(8) : Tews I., Vincentelli R., Vorgias C. E. 1996. N-Acetylglucosaminidase (chitobiase) from Serratia marcescens: gene sequence, and protein production and purification in Escherichia coli. Gene. 170: 63-67.

(9) : Degrees of freedom. Investopedia. [consulted: 30 setember 2017]. Available at: http://www.investopedia.com/terms/d/degrees-of-freedom.asp

(10) : Saranya Peerakietkhajorn, Koji Tsukada, Yasuhiko Kato, Tomoaki Matsuura and Hajime Watanabe. 2015. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, *Daphnia magna.* Environmental Microbiology Reports. 7: 364–372

(11) : Heike M. Freese and Bernhard Schink. 2011. Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean *Daphnia magna*. Microb Ecol. 62: 882.

(12) : Anja Poehlein, Heike M. Freese, Rolf Daniel and Diliana D. Simeonova. 2014. Draft Genome Sequence of Serratia sp. Strain DD3, Isolated from the Guts of *Daphnia magna.* Genome Announc. 2: 14.

(13) : Alexandra A. Mushegian, Dieter Ebert. 2017. Presence of microbiota reverses the relative performance of *Daphnia* on two experimental diets. 125: 29-31.

(14) : Thomas J. Kirn, Brooke A. Jude, Ronal K. Taylor. 2005. A colonization factor links *Vibrio*

cholerae environmental survival and human infection.

(15) : Carl J. Sindermann. 1989. The Shell Disease Syndrome in Marine Crustaceans. NMFS.

(16) : Claire L. Vogan, Carolina Costa-Ramos and Andrew F. Rowley. 2002. Shell disease syndrome in the edible crab, Cancer pagurus – isolation, characterization and pathogenicity of chitinolytic bacteria. Microbiology. 148: 743–754

(17) : May Bente Brurberg, Bjørnar Synstad, Sonja Sletner Klemsdal, Daan M.F. van Aalten, Leif Sundheim and Vincent G.H. Eijsink. 2000. Chitinases from *Serratia marcescens.* Recent Research Developments in Microbiology.

8. Tables

Table 1

	Measure 1	Measure 2	Measure 3	Total
Mean	7.55	7.72	7.83	7.55
Standard deviation (SD)				0.1410674

Outcomes of RStudio concerning the mean and the standard deviation of the pH points of the three water tanks.

Table 2

Bacterium	**Nº of *Daphnia* at the starting point**	**Living *Daphnia* after 24 hours. (1st experiment)**	**Living *Daphnia* after 24 hours. (2nd experiment)**	**Living *Daphnia* after 24 hours. (3rd experiment)**	**Average percentage of mortality (%)**
Bacillus cereus	20	17	15	13	25%
Bacillus megaterium	20	8	11	9	53%
Escherichia coli	20	8	9	8	58%
Micrococcus luteus	20	0	3	5	87%
Pseudomonas fluorescens	20	10	9	11	50%
Staphylococcus epidermidis	20	8	10	7	58%
Serratia marcescens	20	0	0	0	100%

Mortality rate of the 7 different bacteria after 3 experiments for each one. The three infections started with 20 living Daphnia. In red, mortality rates above 75%.

Table 3

Molecule	**Family**	**Uniprot ID**	**PDB ID**	**Resolution (Å)**	**Method**
Chitinase A	GH-18	P07254	1CTN	2.3	X-Ray diffraction
Chitinase B	GH-18	Q54276	1E15	1.9	X-Ray diffraction
Chitinase C	GH-18	Q700B8	4AXN	1.68	X-Ray diffraction
Chitobiase	GH-20	Q54468	1QBA	1.85	X-Ray diffraction
CBP21	CBM33	O83009	2BEM	1.55	X-Ray diffraction

Families and IDs of the chitinolytic machinery of Serratia marcescens. Resolution and Method information is about the PDB entries.

Table 4

Docking	**Shapiro-Wilk normality test**	**Anderson-Darling normality test**	**Cramer-von-Mises normality test**	**Kolmogorov-Smirnov normality test**	**Pearson normality test**	**Shapiro-Francia normality test**
ChiA + Chitin	$3.479 \cdot 10^{-8}$	$1.645 \cdot 10^{-12}$	$8.565 \cdot 10^{-9}$	$2.592 \cdot 10^{-9}$	$1.74 \cdot 10^{-11}$	$2.643 \cdot 10^{-7}$
ChiB + Chitin	$2.257 \cdot 10^{-7}$	0.001661	0.04013	0.07359	$9.495 \cdot 10^{-5}$	$9.486 \cdot 10^{-7}$
ChiC1 + Chitin	$2.811 \cdot 10^{-9}$	$1.468 \cdot 10^{-10}$	$4.886 \cdot 10^{-8}$	$9.853 \cdot 10^{-7}$	$2.221 \cdot 10^{-15}$	$2.439 \cdot 10^{-8}$
Chitobiase + Chitobiose	$5.57 \cdot 10^{-5}$	$2.997 \cdot 10^{-5}$	$7.774 \cdot 10^{-5}$	$3.096 \cdot 10^{-6}$	$1.506 \cdot 10^{-12}$	0.0001317

Results of the different normality tests performed by RStudio with the data obtained from the SwissDock assessment. In black, results confirming that the Energy data has a non-normal distribution. In red, results confirming the normal distribution of the Energy data. A p-value under 0.05 means that data has a non-normal distribution. All these tests are made to distinguish what methods will be applied later depending on their kind of distribution.

Table 5.

***Daphnia*'s health condition**	**Mean of the heart beats counted on 4 different specimens**				**Mean**	**Standard deviation**
Healthy *Daphnia*	200	202	198	195	199	2.58
Infected with *Serratia marcescens*	177	186	174	183	180	5.48

Difference in the heart beats of healthy Daphnias magna and infected Daphnias magna at a temperature of 20ºC. The number of heart beats are counted in periods of 20 seconds and then multiplied by 3. The infected Daphnias were in contact with Serratia marcescens for 8 hours. We can observe that the mean was reduced from 199 beats/minute to 180 beats/minute, that is to say that the heartbeat decreased a 5.25%.

Table 6.

Data	**Shapiro-Wilk normality test**	**Pearson normality test**
Healthy *Daphnia* heart beat	0.9523	1
Infected *Daphnia* heart beat	0.7143	1

Results of the normality tests performed with RStudio. All the results are higher than 0.05, what means that both sets of data have a normal distribution as their p-value is higher than p=0.05.

Table 7.

Data	T-value	Degrees of freedom	P-value
Healthy and Infected *Daphnia* heart beat	6.0112	4.6386	0.002367

Results of the Welch two sample t-test (RStudio).